Catalytic Roles of Yeast GSK3β/Shaggy Homolog Rim11p in Meiotic Activation

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Manuscript received April 23, 1999
Accepted for publication July 14, 1999

ABSTRACT

In *Saccharomyces cerevisiae*, many meiotic genes are activated by a heteromeric transcription factor composed of Ime1p and Ume6p. Ime1p-Ume6p complex formation depends upon the protein kinase Rim11p, which interacts with and phosphorylates both Ime1p and Ume6p *in vitro*. Rim11p may promote complex formation through its phosphorylation of Ime1p and Ume6p or simply through its interaction with both proteins. Here, we characterize mutant Ime1p derivatives that interact with Rim11p but are not phosphorylated *in vitro*. These mutant proteins are also defective in interaction with Ume6p. These results argue that Ime1p must be phosphorylated to interact with Ume6p. Our genetic observations suggest that Ime1p tyrosine residues are among the Rim11p phosphoacceptors, and we find that Ime1p reacts with an anti-phosphotyrosine antibody. Ime1p and Rim11p have been thought to act only through Ume6p, but we find that Ime1p and Rim11p promote meiosis at a very low level in the absence of Ume6p. A nonphosphorylatable mutant Ime1p derivative promotes sporulation through this Ume6p-independent pathway, as does a mutant Rim11p derivative that fails to interact with Ime1p. Therefore, Ime1p and Rim11p have two genetically separable functions in the sporulation program. However, catalytic activity of Rim11p is required for sporulation in the presence or absence of Ume6p.

THE protein kinase Rim11p (also called Mds1p and ScGSK3) is required for meiosis in yeast (Mitchell and Bowdish 1992; Bowdish *et al.* 1994; Puziss *et al.* 1994). Rim11p promotes formation of the Ime1p-Ume6p complex (Rubin-Bejerano *et al.* 1996), which activates transcription of early meiotic genes (reviewed in Kupiec *et al.* 1997). Fusion of a transcriptional activation domain to Ume6p permits meiosis in the absence of Rim11p and Ime1p (Rubin-Bejerano *et al.* 1996), and so the only essential role in meiosis of Rim11p and Ime1p is apparently to modify Ume6p.

Rim11p is similar to members of the eukaryotic glycogen synthase kinase-3β (GSK3β)/shaggy family in both structure and function (Cadigan and Nusse 1997). GSK3β family members have catalytic regions with >80% amino acid sequence identity and share 55–60% identity with Rim11p. (For comparison, the GSK3β and protein kinase A catalytic domains share <25% identity.) Several of the GSK3β family members act in the *Wnt*/wingless signaling pathway to promote formation of a β-catenin-adenomatous polyposis coli (APC) complex (Cadigan and Nusse 1997). The Ime1p-Ume6p and β-catenin-APC complexes have no structural similarity, but their relationships to Rim11p and GSK3β share a common feature: the protein kinases bind to and phosphorylate both subunits of their target complex (Rim11p, Bowdish *et al.* 1994; Malathi *et al.* 1997; GSK3β, Rubinfeld *et al.* 1996; Behrens *et al.* 1998; Ikeda *et al.* 1998; Yamamoto *et al.* 1998). This finding is consistent with two distinct models for Rim11p and GSK3β function. One is that the kinases have a catalytic role in complex formation; for example, Rim11p phosphorylates both Ime1p and Ume6p, and phospho-Ime1p then interacts with phospho-Ume6p (Figure 1A). Numerous other protein kinases act catalytically. The other model is that the kinases have a structural role; for example, a Rim11p-Ime1p complex interacts with Ume6p, and the functional transcriptional activator may be a ternary Ime1p-Rim11p-Ume6p complex (Figure 1B). Pbs2p and Kss1p are examples of protein kinases that have structural roles (as well as catalytic roles) in signal transduction (Posas and Saito 1997; Bardwell *et al.* 1998).

Properties of *rim11* and *ume6* mutants are consistent with either catalytic or structural roles for Rim11p (Malathi *et al.* 1997; K. Malathi and A. P. Mitchell, unpublished results). One observation is that two catalytically defective mutant Rim11p derivatives fail to promote Ime1p-Ume6p complex formation, as predicted by the catalytic model. However, both Rim11p derivatives are also defective in interaction with Ume6p (and with Ime1p). A second observation is that the mutant Ume6-T99Np, which has a substitution that reduces its phosphorylation *in vitro*, fails to interact with Ime1p, as predicted by the catalytic model. However, Ume6-T99Np is also defective in interaction with Rim11p. So, in both cases, the defects in Ime1p-Ume6p complex formation are predicted by the structural model as well. Thus, it has not been possible to use the Rim11p-Ume6p

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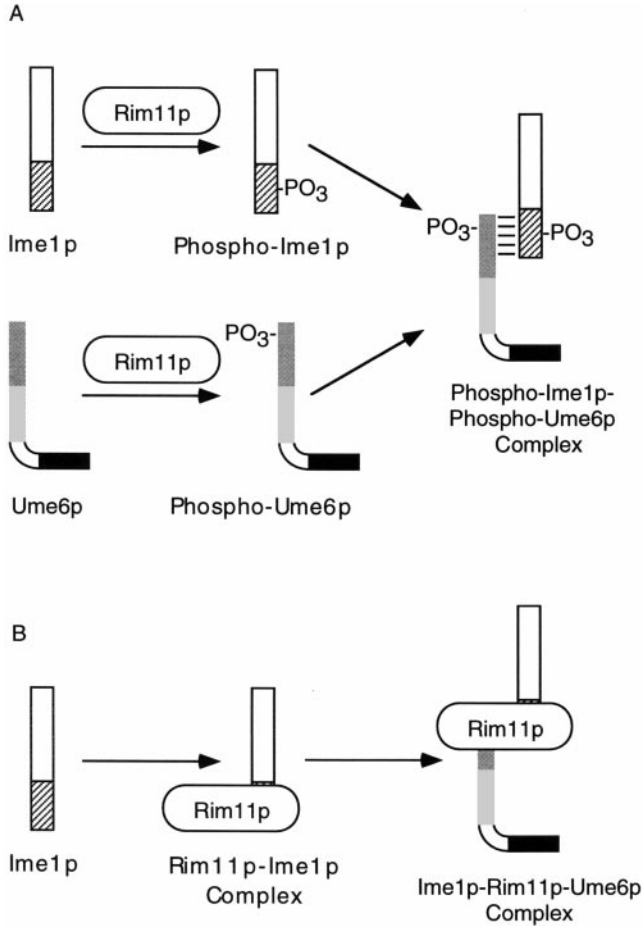

Figure 1.—Possible catalytic and structural roles for Rim11p. (A) Catalytic model. Rim11p phosphorylates Ime1p and Ume6p, then phospho-Ime1p binds to phospho-Ume6p. (B) Structural model. Rim11p binds first to Ime1p, then the Rim11p-Ime1p complex binds to Ume6p. (It is equally possible that Rim11p binds first to Ume6p, then the Rim11p-Ume6p binds to Ime1p.)

complex to distinguish between catalytic and structural roles for Rim11p, because the mutations we have examined affect both catalytic and structural attributes.

Here, we examine the effects of *ime1* mutations on both Rim11p-Ime1p and Ime1p-Ume6p complexes. Their properties support a catalytic role for Rim11p in promoting Ime1p-Ume6p complex formation. In addition, our analysis indicates that Ime1p and Rim11p have a second role in promoting meiosis that is independent of Ume6p.

MATERIALS AND METHODS

Yeast strains: Strains used in this study (Table 1) were constructed through genetic crosses and transformations, following standard procedures (Kaiser *et al.* 1994). All strains derive from crosses among SK-1 strains (Kane and Roth 1974; Alani *et al.* 1987) except strains Y187 and Y190 (Durfee *et al.* 1993), which were used for two-hybrid interaction cloning. The *ume6Δ::TRP1* mutation was created by PCR product-directed gene disruption (Baudin *et al.* 1993; Lorenz *et al.* 1995) through amplification of the *TRP1* cassette from plasmid pRS314 with primers UME6-1050 and UME6-3′-3590. The mutation removes *UME6* sequences between 18 bp upstream of the initiation codon and 102 bp upstream of the termination codon.

***P*$_{GAL1}$*-IME1Δ68-182* mutants:** P_{GAL1}*-IME1Δ68-182* internal deletions of previously isolated random P_{GAL1}*-IME1* mutants (Smith *et al.* 1993) were constructed as follows. Plasmid pHS136 (YCpP_{GAL1}*-IME1*) was digested with *Pvu*II and *Sac*I to release the 3′ portion of the *IME1* open reading frame (ORF) and then ligated to 1.0-kb *Eco*RV/*Sac*I fragments carrying the various *ime1* mutations.

Several oligonucleotides (see sequences below) were used to create *IME1* mutations. To construct site-directed alanine substitutions, plasmid pKM153 (P_{GAL1}*-IME1Δ68-182* in vector pRS316) was mutagenized with oligonucleotides S1 (to create S352A), S2 (to create S356A), and S3 (to create S360A). The multi-site "ala8" replacement mutant was generated by PCR using oligonucleotides IM1C and YX3 with plasmid pKM153 as a template. A corresponding wild-type plasmid was constructed in a PCR with oligonucleotides M127 and YX3. The PCR products were digested with *Hin*dIII and *Sal*I and cloned into *Hin*dIII- and *Sal*I-digested plasmid pSV150, which is vector pRS316 carrying the *GAL1* promoter (Vidan and Mitchell 1997). The resulting plasmids carried P_{GAL1}*-IME1Δ68-182*, but lacked *IME1* 3′ noncoding sequences. Ime1p was expressed poorly from these plasmids; expression was improved by insertion of *IME1* 3′ noncoding sequences, cloned into the plasmids' *Sal*I site from a PCR with oligonucleotides M128 and M129. The sequence of the entire P_{GAL1}*-IME1Δ68-182-ala8* ORF was verified.

Two-hybrid interaction assays: Overnight cultures in SC-Trp-Leu medium were diluted into yeast extract peptone acetate (YPAc) and harvested after two to three doublings (12–14 hr). β-Galactosidase assays were performed on permeabilized cells (Bowdish and Mitchell 1993). Each determination is an average of three independent transformants. The range was <20% of the mean for values over 150 Miller units and 25% of the mean for values under 150 Miller units. Transformants of strains Y190 and Y187 were mated for use in these assays.

Plasmids specifying Gal4p DNA binding domain (GBD)-Rim11p, GBD-Ime1p(294–360), and Gal4p activation domain (GAD)-Ume6p(1–161) have been described previously (Malathi *et al.* 1997). Plasmids specifying GAD-Ime1p and derivatives were constructed as follows. PCR amplification from P_{GAL1}*-IME1* templates with oligonucleotides IM1N and IM1B created an *Nco*I site at codon 1 and a *Bam*HI site at a location 200 bp downstream of the *IME1* stop codon. PCR products digested with *Nco*I and *Bam*HI were cloned into *Nco*I- and *Bam*HI-digested plasmid pACTII (Durfee *et al.* 1993).

Plasmids specifying GBD-Ime1p(294–360) mutant derivatives were generated by *in vivo* homologous recombination (Ma *et al.* 1987). PCR amplification of *IME1* plasmids with oligonucleotides M132 and M133 created products in which *IME1* codons 294–360 and 200 bp of 3′ noncoding sequences were flanked by pAS1-CYH2 polylinker sequences, arranged to create an in-frame fusion. Purified PCR products were co-transformed into strain Y187 along with plasmid pAS1-CYH2 digested with *Nco*I and *Bam*HI. Trp^+ transformants were tested for expression of GBD-fusion protein by immunoblotting with anti-Ime1p and plasmids were retrieved in *Escherichia coli*. Presence of the mutations was confirmed by sequencing.

Synthetic oligonucleotides: Synthetic oligonucleotides used in these studies had 5′ to 3′ sequences indicated in parentheses: IM1C (AGT ACT TGT CGA CAA TTA AGC AGC GGC TTT AGC AAA CTT GGC GGC TAT TTC TTG AAA CCT GAC CTT GTC AGC AGC ATC TTG ATC ATT AGA ACT GCT G), M127 (AGT ACT TGT CGA CAA TTA AGA ATA GGT TTT ACT), M128 (CTT AAT TGT CGA CAA GTA CTA

TABLE 1

Yeast strains

Strain[a]	Genotype[b]
KB267	**a**/α *rim11::LEU2/rim11::LEU2 ime2Δ4-lacZ::LEU2/+ met4/+ +/his4*
M1085	**a**/α *RIM11-HA/RIM11-HA ime1Δ12::TRP1/ime1Δ12::TRP1 met4/+ +/his3*
M1121	**a**/α *RIM11-HA/RIM11-HA ime1Δ12::TRP1/ime1Δ12:TRP1 ime2Δ4-lacZ::LEU2/+ met4/+ +/his4*
YX282	**a**/α *ime1Δ20/ime1Δ20 ume6Δ::TRP1/ume6Δ::TRP1*
YX306	**a**/α *ume6Δ::TRP1/ume6Δ::TRP1*
YX471	**a**/α *rim11:LEU2/rim11::LEU2 ume6Δ::TRP1/ume6Δ::TRP1*
Y187	α *gal4 gal80 his3 trp1-901 ade2-101 leu2-3, 112 ura3-52::URA3-GAL1-lacZ*
Y190	**a** *gal4 gal80 his3 trp1 ade2 ura3 leu2 URA3::GAL1-lacZ LYS2::GAL1-HIS3 cyh^r*

[a] All strains except Y187 and Y190 are derived from strain SK-1.
[b] All haploid SK-1 derivatives have additional markers *ura3 leu2 try1 lys2 ho::LYS2 gal80::LEU2*, and diploid SK-1 derivatives are homozygous for those markers.

CAA TC), M129 (CGA TAA GGG TAC CAA CGC TAC GGT A), M132 (GGT CAA AGA CAG TTG ACT GTA TCG CCG GTA TTG CAA TAC CCA GCT TTG ACT CAT ATG GCC ATG GTA GAA ATT GCC TTC GAC GTT GAA), M133 (GTT ACT CAA GAA CAA GAA TTT TCG TTT TAA AAC CTA AGA GTC ACT TTA AAA TTT GTA TAC ACT TAT GGA TCC TAA TAA CGC TAC GGT ATT ATG), IM1N (GGC ATG CCA TGG AGC AAG CGG ATA TGC ATG G), IM1B (CGA TAA GGA TCC TAA TAA CGC TAC GG), UME6-1050 (GAA GCG CCC ACC TTC GCA CAG CGC ACA GGA ACT AGG ACA CTA CCG CAC TCA AAC CAT TTG GCA GAT TGT ACT GAG AGT GC), UME6-3′-3590 (GAT TTC CTC CAG TTT CAT CTG TTT TTT CTT TGG ATC AGA TAC AAA ATC TGG TTT GAA CGC CGC ATC TGT GCG GTA TTT CAC), YX3 (CAG CGA AGC GAT GAT TTT TGA TC), S1 (GGT TTC AAG AAA TAG CCT ACA AAG), S2 (CCT ACA AGT TTG CTA AAA CCT AT), S3 (TAA AAC CTA TGC TTA ATT CTC G).

Miscellaneous: Procedures for Rim11-HAp immunoprecipitation, immunoblotting, protein kinase activity assays, and measurement of sporulation have been described (Bowdish *et al.* 1994; Malathi *et al.* 1997). For anti-phosphotyrosine immunoblots, filters were blocked for 2 hr with PyTBST buffer (100 mm Tris pH 8.0, 750 mm NaCl, 1% Tween 20, 10 mm EDTA) containing 3% bovine serum albumin and 1% ovalbumin. The filter was then probed for 16 hr at 4° with mouse monoclonal antibody 4G10 (Upstate Biotechnology) diluted 1/1000 in the blocking solution, washed three times with PyTBST buffer, then incubated with 1/3000 diluted secondary antibody and ECL reagents as described previously (Malathi *et al.* 1997). For immunoprecipitation experiments with strains expressing the Ime1Δ68-182-ala8p, we used 4 mg of protein extract and 4.0 μl of 5.5 mg/ml 12CA5 monoclonal antibody followed by a 2-hr incubation with 40 μl of 50% protein A Sepharose beads. Levels of the mutant Ime1Δ68-182-ala8p were much lower than Ime1Δ68-182p in crude extracts. Therefore, we used plasmid pKM212, which specifies Ime1Δ68-182p but lacks *IME1* 3′ noncoding sequences, to express Ime1Δ68-182p at reduced levels as a positive control. The multi-copy *RIM11-HA* plasmids pKM116 (*RIM11-HA*), pKM117 (*RIM11-HA-K68R*), pKM118 (*RIM11-HA-Y199F*), and pKM119 (*RIM11-HA-K68A*) resulted from transfer to vector pRS423 of the inserts from plasmids pKB166, pKB171, pKB201, and pKB199, respectively (Bowdish *et al.* 1994).

RESULTS

Interaction of Rim11p with Ime1p mutant derivatives: Rim11p and Ime1p form a complex in which Ime1p is phosphorylated. To determine whether interaction or phosphorylation is required for Ime1p function, we have characterized Rim11p interaction with and phosphorylation of Ime1p mutant derivatives.

Complex formation was assayed through co-immunoprecipitation of Ime1p with epitope-tagged Rim11-HAp (Figure 2, A and B) and through two-hybrid interaction assays (Table 2). For coimmunoprecipitation experiments, *ime1* missense mutations were introduced into a functional Ime1Δ68-182p internal deletion derivative (Smith *et al.* 1993) because Ime1p comigrates with

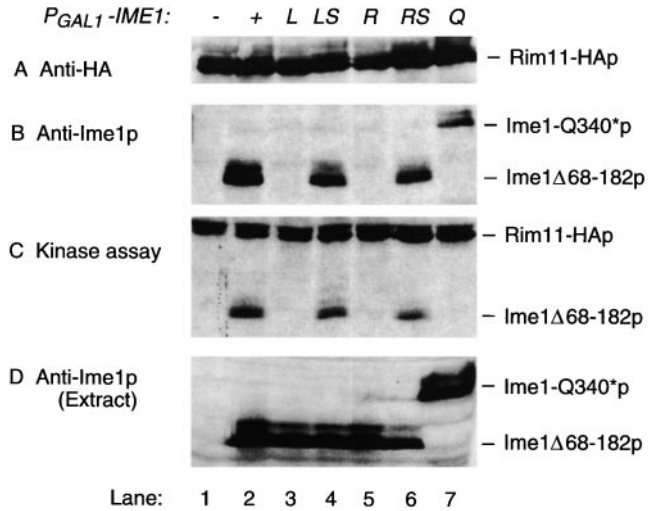

Figure 2.—Effects of *ime1* mutations on Rim11p-Ime1p complex formation and phosphorylation. Anti-HA immune complexes were prepared from strain M1085 (genotype, *RIM11-HA/RIM11-HA ime1Δ/ime1Δ*) carrying plasmids YCpP_{GAL1} (lane 1), YCpP_{GAL1}-*IME1Δ68-182* (lane 2), YCpP_{GAL1}-*IME1Δ68-182-L321F* (lane 3), YCpP_{GAL1}-*IME1Δ68-182-L321F,S360F* (lane 4), YCpP_{GAL1}-*IME1Δ68-182-R347K* (lane 5), YCpP_{GAL1}-*IME1Δ68-182-R347K,S360F* (lane 6), or YCpP_{GAL1}-*IME1-Q340** (lane 7) after growth in YPAc medium. The immune complexes were split: half was used in an immunoblot with anti-HA monoclonal antibody (A) and anti-Ime1p antiserum (B); half was used in protein kinase assays (C). Presence of Ime1p derivatives in crude extracts was determined through an immunoblot with anti-Ime1p antiserum (D).

TABLE 2

Interaction between Rim11p and mutant Ime1p derivatives

GAD fusion[a]	Two-hybrid interaction with GBD-Rim11p[b] (β-galactosidase activity)[c]
None	0.12
Ime1p	2300
Ime1-L321Fp	175
Ime1-R347Kp	193
Ime1-Q340*p	904
Ime1-E294*p	0.1
Ime1-L321F, S360Fp	833
Ime1-R347K, S360Fp	815

[a] GAD-Ime1p has residues 1 to 360 of Ime1p.
[b] GBD-Rim11p has residues 1 to 370 of Rim11p.
[c] Strain Y190 was used in these assays. β-Galactosidase activity was determined in log phase cultures in SC-TRP-Leu medium. The values, in Miller units, are the mean of three determinations; all standard deviations were <25%.

Rim11p on SDS-PAGE. Ime1Δ68-182p was detectable in Rim11-HAp immune complexes (Figure 2, lane 2 compared to lane 1). Mutant products Ime1Δ68-182-L321Fp and Ime1Δ68-182-R347Kp were not detectable in Rim11-HAp immune complexes (Figure 2, lanes 3 and 5), although they were present in crude extracts (Figure 2D, lanes 3 and 5). Similarly, the L321F and R347K substitutions caused a 12-fold Ime1p-Rim11p interaction defect in two-hybrid assays (Table 2). The S360F second-site substitution restores functional activity to L321F and R347K mutants (Smith *et al.* 1993) and restores considerable ability to bind to Rim11p (Figure 2, lanes 4 and 6, and Table 2). The nonsense mutant product Ime1-Q340*p was detectable in Rim11-HAp immune complexes (Figure 2, lane 7) and had 2.5-fold decreased interaction with Rim11p in two-hybrid assays (Table 2). Therefore, the L321F and R347K substitutions cause a severe defect in Ime1p-Rim11p interaction and the Q340* truncation causes a mild defect.

The ability of each mutant Ime1p derivative to serve as a phosphorylation substrate was assayed in Rim11-HAp immune complexes (Figure 2C). Phosphorylation of Ime1Δ68-182p was detected (lane 2, compared to lane 1). Phosphorylation of the L321F and R347K mutant proteins was not detected (lanes 3 and 5). This result was expected from their poor recovery in immune complexes, and the S360F second-site substitution restored phosphorylation of both mutant proteins (lanes 4 and 6). Phosphorylation of Ime1-Q340*p was not detected (lane 7). The Ime1-Q340*p extract did not contain an inhibitor of Rim11-HAp protein kinase activity, because autophosphorylation of Rim11-HAp was detectable at similar levels in all extracts (Figure 2C). Thus the Q340* truncation permits Rim11p-Ime1p binding, but prevents Ime1p from serving as a substrate for Rim11p phosphorylation *in vitro.*

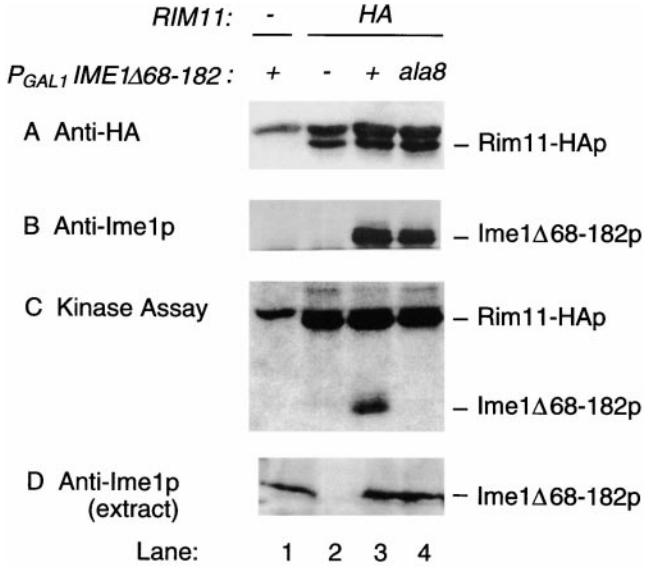

Figure 3.—Effects of the ala8 substitution on Rim11p-Ime1p complex formation and phosphorylation. Anti-HA immune complexes were prepared from strains KB267 (genotype, *rim11/rim11*; lane 1) and M1085 (genotype: *RIM11-HA/RIM11-HA;* lanes 2–4) carrying plasmids pKM212 (YCpP_{GAL1}-*IME1Δ68-182* lacking 3′ noncoding sequences; lanes 1 and 3), YCpP_{GAL1} (lane 2), or YCpP_{GAL1}-*IME1Δ68-182-ala8* (lane 4) after growth in YPAc medium. As in Figure 2, the immune complexes were split: half was used in an immunoblot with anti-HA monoclonal antibody (A) and anti-Ime1p antiserum (B); half was used in protein kinase assays (C). Presence of Ime1p derivatives in crude extracts was determined through an immunoblot with anti-Ime1p antiserum (D).

Identification of putative phosphoacceptor residues in Ime1p: The studies above suggest that amino acid residues beyond Q340 of Ime1p are required for Ime1p phosphorylation by Rim11p. Therefore, the eight serine, threonine, and tyrosine residues distal to Q340 in Ime1Δ68-182p are candidate sites of phosphorylation. To test this hypothesis, we examined the Ime1Δ68-182-ala8p mutant derivative, in which these eight residues were replaced with alanines. Ime1Δ68-182-ala8p accumulates to lower levels than wild-type Ime1Δ68-182p (data not shown). Therefore, we used wild-type Ime1Δ68-182p expressed at reduced levels, due to a deletion of 3′ noncoding sequences, as a standard for comparison with Ime1-ala8p. These two proteins were present at comparable levels in crude extracts (Figure 3D, lanes 3 and 4) and were recovered at similar levels in Rim11-HAp immune complexes (Figure 3B). However, Rim11-HAp immune complexes phosphorylated Ime1Δ68-182-ala8p very poorly compared to Ime1Δ68-182p (Figure 3C). These results indicate that some or all of the serine, threonine, and tyrosine residues in Ime1p distal to Q340 are required for phosphoacceptor activity *in vitro.*

The serine residues S352, S356, and S360 match the GSK3 substrate site consensus S-X_3-S-X_3-S (Roach 1991). To determine whether these residues are the main Ime1p phosphoacceptors, we characterized mu-

TABLE 3

Functional analysis of Ala replacement mutants

Ime1p derivative[a]	*ime2-lacZ* expression[b] (Miller units)	Sporulation[b] (%)	Phosphorylation
No Ime1P	1.1	<0.1	−
Ime1Δ68-182P	196	73	+
Ime1Δ68-182-Q340*p	4.0	1.0	−
Ime1Δ68-182-S352Ap	43	33	+
Ime1Δ68-182-S356Ap	86	28	+
Ime1Δ68-182-S360Ap	167	46	+
Ime1Δ68-182-S352A, S356A, S360Ap	62	30	+
Ime1Δ68-182p (low expression)[c]	148	65	+
Ime1Δ68-182-ala8p	2.0	<0.1	−

[a] Ime1p derivatives are all expressed from the *GAL1* promoter. Ime1Δ68-182-ala8p has substitutions Y342A, Y343A, S352A, Y353A, S356A, T358A, Y359A, and S360A.

[b] Strain M1121 (*ime1Δ/ime1ΔIME2/ime2-lacZ*) carrying the indicated P_{GAL1}-*IME1Δ68-182* plasmids was incubated in sporulation medium for determination of β-galactosidase activity (after 8 hr) and sporulation ability (after 24–72 hr).

[c] Ime1Δ68-182p was expressed at reduced levels from plasmid pKM212, which lacks *IME1* 3′ noncoding sequences.

tants in which these serines were replaced with alanines. All single and multiple mutant Ime1p derivatives were present at similar levels in crude extracts and in Rim11-HAp immune complexes, and all mutant Ime1p derivatives were phosphorylated in immune complexes (Table 3 and data not shown). Notably, the triple substitution eliminating all three serine residues had little effect on Ime1p phosphorylation. Thus the Ime1p residues in this consensus GSK3 site are not required for phosphoacceptor activity *in vitro*.

We considered the hypothesis that Rim11p phosphorylates Ime1p on one of the tyrosine residues (Y342, Y343, Y353, and Y359) among residues 340–360. To test this model, we assayed reactivity of Ime1Δ68-182p, after its phosphorylation in Rim11-HAp immune complexes, with an anti-phosphotyrosine antibody. We detected a protein that reacts with the antibody (Figure 4A, lane 4), which has an electrophoretic mobility similar to that of Ime1Δ68-182p. Presence of the protein depends upon expression of both Ime1Δ68-182p and Rim11-HAp (Figure 4A, lanes 3 and 1, respectively). Therefore, we believe that the protein is Ime1Δ68-182p. We detected no reaction of Ime1Δ68-182-ala8p with the antibody (Figure 4, A and B, lane 5). These results support the hypothesis that Rim11p phosphorylates Ime1p on tyrosine residues.

Functional analysis of Ime1p mutant derivatives: Functional activity of new Ime1p mutant derivatives was assessed through their abilities to activate *ime2-lacZ* expression and promote sporulation (Table 3). Ime1Δ68-182-ala8p was completely defective in both assays. Reduced accumulation of the mutant protein could not account for the defect, because a reduction in expression of Ime1Δ68-182p caused only a mild reduction in *ime2-lacZ* expression and sporulation (Table 3). Prior studies indicated that Ime1-Q340*p had reduced activity (Smith *et al.* 1993), and we found a slightly more severe defect in assays of Ime1Δ68-182-Q340*p (Table 3). Properties of these mutants argue that interaction of Ime1p with Rim11p is not sufficient for full functional activity; phosphorylation of Ime1p is required as well.

Relationship between Ime1p phosphorylation and Ime1p-Ume6p interaction: To determine whether Ime1p phosphorylation may be required for Ime1p-Ume6p interaction, we carried out two-hybrid interaction assays with GBD-Ime1p mutant derivatives and

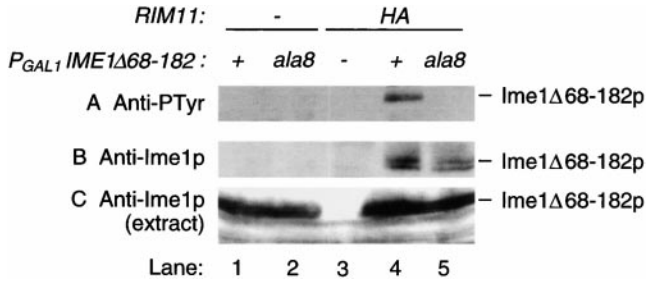

Figure 4.—Reactivity of Ime1p with anti-phosphotyrosine monoclonal antibody. Anti-HA immune complexes were prepared from strains KB267 (genotype, *rim11/rim11*; lanes 1 and 2) and M1085 (genotype, *RIM11-HA/RIM11-HA;* lanes 3–5) carrying plasmids pKM212 (YCpP_{GAL1}-*IME1Δ68-182* lacking 3′-noncoding sequences; lanes 1 and 4), YCpP_{GAL1}-*IME1Δ68-182-ala8* (lanes 2 and 5), or YCpP_{GAL1} (lane 3) after growth in YPAc medium. The immune complexes were incubated for 20 min under immune complex kinase assay conditions in the presence of 0.02 mm unlabeled ATP (and without radiolabeled ATP). The immune complexes were then split to use in immunoblots with anti-phosphotyrosine monoclonal antibody (A) and with anti-Ime1p antiserum (B). Presence of Ime1p derivatives in crude extracts was determined through an immunoblot with anti-Ime1p antiserum (C).

TABLE 4

Effect of *ime1* mutations of Ime1p-Ume6p interaction

GBD-fusion[a]	Two-hybrid interaction with GAD-Ume6p[b] (β-galactosidase activity[c])
None	0.77
Ime1p	422
Ime1-L321Fp	29
Ime1-R347Kp	1.5
Ime1-Q340*p	2.8
Ime1-L321F, S360Fp	221
Ime1-R347K, S360Fp	42
Ime1-S352A, S356A, S360Ap	214
Ime1-ala8p	0.4

[a] GBD-Ime1p includes residues 294–360 from Ime1p. GBD-Ime1p derivatives yielded background β-galactosidase levels of 0.2 to 1.2, as determined with GAD lacking Ume6p residues.
[b] GAD-Ume6p includes residues 1–161 from Ume6p.
[c] Strain Y190 was used in these assays. β-Galactosidase activity was determined in log phase cultures in YPAc medium. The values, in Miller units, are the mean of three determinations and had standard deviations of <30% (for values <200 units) or <20% (for values >200 units).

GAD-Ume6p (Table 4). All GBD-Ime1p fusion proteins were detected on immunoblots probed with anti-Ime1p antiserum, although the wild-type fusion protein accumulated to levels approximately fivefold higher than several of the mutant fusion proteins (data not shown). Ime1p substitutions that impair binding to Rim11p (L321F and R347K), as well as those that permit Rim11p binding but impair phosphorylation (Q340* and ala8), all caused reduced interaction with Ume6p. The second-site substitution that improves Rim11p binding and phosphorylation (S360F) improved interaction between mutant Ime1p derivatives and Ume6p. In summary, alterations of Ime1p that result in phosphorylation defects also result in Ume6p interaction defects. These findings support the hypothesis that Ime1p phosphorylation by Rim11p, not simply its association with Rim11p, is required for Ime1p-Ume6p interaction.

Genetic evidence for an additional function of Ime1p and Rim11p: It is well established that Ime1p and Rim11p act in conjunction with Ume6p to activate early meiotic genes. However, *ime1* and *rim11* mutants are unable to sporulate, whereas *ume6* mutants are able to sporulate weakly (Bowdish and Mitchell 1993; Strich *et al.* 1994; Steber and Esposito 1995). Thus it seemed possible that Ime1p and Rim11p might have an additional role in sporulation. We tested this hypothesis through assays of sporulation in *ume6*Δ mutant strains (Table 5). A *UME6/UME6* diploid yielded 93% sporulation; an isogenic *ume6*Δ/*ume6*Δ diploid yielded 3.2% sporulation. Homozygous null *ime1*Δ or *rim11*Δ mutations abolished sporulation in *UME6/UME6* and *ume6*Δ/*ume6*Δ backgrounds. These observations indicate that Ime1p and Rim11p promote sporulation through a second mechanism that is independent of Ume6p.

To determine whether Ume6p-independent sporulation depends upon Ime1p phosphorylation, we assayed several Ime1p mutant derivatives for their ability to stimulate sporulation in an *ime1*Δ *ume6*Δ homozygous diploid (Table 5). Expression of Ime1Δ68-182p yielded 4.9% sporulation. Sporulation was reduced only twofold by the Q340* substitution. Sporulation was abolished by the ala8 substitution. Reduced protein accumulation may have contributed to the ala8 defect, because reduced expression of Ime1Δ68-182p caused a fourfold reduction in sporulation ability. Functional activity of Ime1Δ68-182-Q340*p in this assay argues that the phosphorylation of Ime1p assayed *in vitro* is not required for Ume6p-independent sporulation.

To determine whether Ume6p-independent sporulation depends upon Rim11p protein kinase activity, we assayed Rim11p mutant derivatives for ability to stimulate sporulation in a *rim11*Δ *ume6*Δ homozygous diploid (Table 5). Expression of Rim11-HAp yielded 4.8% sporulation. Sporulation was abolished by K68R and K68A substitutions, which reduce or abolish protein kinase activity (Bowdish *et al.* 1994). Sporulation was reduced only twofold by the Y199F substitution, which does not abolish Rim11p autophosphorylation activity but causes a severe defect in phosphorylation of Ime1p and Ume6p *in vitro* (Bowdish *et al.* 1994; Malathi *et al.* 1997). These observations support the hypothesis that phosphorylation of Ime1p is not required for Ume6p-independent sporulation. However, they argue that Rim11p protein kinase activity is required for Ume6p-independent sporulation.

DISCUSSION

Prior studies have shown that Rim11p is required for Ime1p-Ume6p complex formation (Rubin-Bejerano *et al.* 1996), and several observations have suggested that Ime1p and Rim11p activate meiosis primarily through formation of an Ime1p-Ume6p complex. In this article, we provide correlative evidence that Rim11p must phosphorylate Ime1p to promote Ime1p-Ume6p complex formation. In addition, we provide genetic evidence that Ime1p and Rim11p each promote sporulation through a second mechanism that is independent of Ume6p.

Function of Rim11p in Ime1p-Ume6p interaction: Rim11p interacts with both Ime1p and Ume6p, and so it has been uncertain whether Rim11p plays structural or catalytic roles in the Ime1p-Ume6p complex. Here, we have characterized two mutant Ime1p derivatives—Ime1-Q340*p and Ime1-ala8p—that bind to Rim11p but undergo no detectable phosphorylation *in vitro*. Both mutants are also defective for interaction with Ume6p. Therefore, our findings argue that interaction between Rim11p and Ime1p is not sufficient to promote Ime1p-Ume6p interaction. The evidence does not rule out

TABLE 5

Sporulation of *ume6*Δ strains

Relevant genotype[a]	Plasmid-specified gene product[b]	Sporulation (%)[c]
IME1 RIM11 UME6	No plasmid	93
*ime1*Δ*RIM11 UME6*	No plasmid	<0.2
*IME1 rim11*Δ *UME6*	No plasmid	<0.2
*IME1 RIM11 ume6*Δ	No plasmid	3.2
*ime1*Δ *RIM11 ume6*Δ	No plasmid	<0.2
*IME1 rim11*Δ *ume6*Δ	No plasmid	<0.2
*ime1*Δ *RIM11 ume6*Δ	Vector	<0.2
*ime1*Δ *RIM11 ume6*Δ	Ime1Δ68-182p	4.9
*ime1*Δ *RIM11 ume6*Δ	Ime1Δ68-182-Q340*p	2.4
*ime1*Δ *RIM11 ume6*Δ	Ime1Δ68-182p (low expression)	1.3
*ime1*Δ *RIM11 ume6*Δ	Ime1Δ68-182-ala8p	<0.2
*IME1 rim11*Δ *ume6*Δ	Rim11-HAp	4.8
*IME1 rim11*Δ *ume6*Δ	Rim11-HA-K68Ap	<0.2
*IME1 rim11*Δ *ume6*Δ	Rim11-HA-K68Rp	<0.2
*IME1 rim11*Δ *ume6*Δ	Rim11-HA-Y199Fp	2.1

[a] All strains are **a**/α diploids and are homozygous for the mutations listed.
[b] Ime1p derivatives were all expressed from the *GAL1* promoter. Rim11p derivatives were expressed from the multicopy plasmids pKM116, pKM117, pKM118, and pKM119.
[c] Patches of each strain were incubated on sporulation plates for 3–4 days at 30°. Sporulation was determined through microscopic examination of at least 300 cells from at least three independent transformants or isolates of each genotype. The range of values was within 25% of each mean.

the possibility that Rim11p remains associated with the Ime1p-Ume6p complex; in fact, the stability of Rim11p-Ime1p *in vitro* leads us to favor this idea (K. Malathi and A. P. Mitchell, unpublished results). However, our observations support the hypothesis that Rim11p must phosphorylate Ime1p to promote Ime1p-Ume6p complex formation (Figure 1A).

It is possible that Ime1p residues 340–360 include the Rim11p phosphorylation sites, because this region is required for phosphoacceptor activity *in vitro*. It includes a GSK3 consensus site (Roach 1991) and a group of tyrosine residues. These residues are all plausible phosphoacceptors for Rim11p, because rat GSK3β is a mixed specificity protein kinase *in vitro* (Wang *et al.* 1994). The consensus site is clearly not the only site of phosphorylation because Ime1-S352A,S356A,S360Ap has significant activity *in vivo* and is a Rim11p substrate *in vitro*. In fact, the consensus site residue S360 has the opposite properties from those expected for a Rim11p phosphoacceptor, because the S360F substitution suppresses Ime1p defects. Thus, if the 340–360 interval is the sole phosphorylated region, then phosphorylation must occur on nonconsensus residues. This conclusion is further substantiated by reactivity of Ime1p with an anti-phosphotyrosine antibody. We believe that the simplest interpretation of our results is that Rim11p phosphorylates Ime1p on one or more of the tyrosine residues Y342, Y343, Y353, and Y359.

It is also possible that Ime1p residues 340–360 act as a regulatory domain that stimulates Rim11p to phosphorylate Ime1p elsewhere. Indeed, suppression of Ime1p mutant defects by the S360F substitution is consistent with a regulatory role for this region.

Genetic characterization of Ume6p-independent sporulation: Null *ume6* mutations relieve repression of early meiotic genes, and so it has seemed likely that sporulation of *ume6* null mutants would be independent of Rim11p and Ime1p (Bowdish and Mitchell 1993; Strich *et al.* 1994; Bowdish *et al.* 1995; Steber and Esposito 1995). However, we have shown in this report that Ime1p and Rim11p are required for sporulation in a *ume6*Δ homozygote. The *ume6*Δ allele cannot specify a partially functional product, because it removes all of the *UME6* ORF except for the most 3′ 34 codons. Therefore, Ime1p and Rim11p must have at least two functions in sporulation: they act in conjunction with Ume6p and independently of Ume6p.

Analysis of Ime1p and Rim11p mutant derivatives in Ume6p-independent sporulation supports the hypothesis that each protein has two distinct functions. For Ime1p, the Q340* truncation severely reduces Ume6p-dependent sporulation but not Ume6p-independent sporulation. The simplest interpretation is that phosphorylation of Ime1p by Rim11p is required only for Ime1p-Ume6p complex formation, and not for the second function of Ime1p. Similarly, for Rim11p, the Y199F substitution abolishes Ume6p-dependent sporulation but not Ume6p-independent sporulation. This Rim11p mutant derivative has a severe defect in interaction with Ime1p, as expected from the location of the substitution in a likely substrate-binding subdomain (Bowdish *et al.* 1994; Malathi *et al.* 1997). This observation suggests

that Rim11p may promote Ume6p-independent sporulation without interacting with Ime1p, or perhaps through a different kind of interaction with Ime1p.

We do not know what additional roles Ime1p and Rim11p may have in meiosis. The idea that Ime1p acts later in meiosis has been proposed by Shefer-Vaida *et al.* (1995), based upon temperature-shift studies of the *ime1-3* mutant. We note that Ume6p is required later in meiosis to reestablish repression of the same early meiotic genes that it activates in conjunction with Ime1p and Rim11p (Bowdish *et al.* 1995; Steber and Esposito 1995). Thus a simple model is that Ime1p, Rim11p, and Ume6p first act together to turn early meiotic genes on, then Ime1p and Rim11p carry out their additional functions while Ume6p acts to turn early meiotic genes off.

We are grateful to members of this laboratory for many helpful discussions and particularly to Teresa Lamb for comments on this manuscript. This work was supported by funds from the National Institutes of Health (GM-39531).

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Communicating editor: A. G. Hinnebusch